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(57) Abstract

Methods are provided for deriving and propagating passagable pluripotent stem cells from early primate embryos. The passagable pluripotent stem cells are capable of differentiating into a variety of intermediate stem cell types through modified *in vitro* culture conditions, and as such are useful as a model for early development, and for treatment of a variety of genetic and degenerative diseases and for assay compounds to determine their effect on development.

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IN VITRO DERIVATION AND CULTURE OF PRIMATE PLURIPOTENT STEM CELLS AND THERAPEUTIC USES THEREOF

FIELD OF THE INVENTION

The field is isolation and culture of primate pluripotent stem cells.

BACKGROUND OF THE INVENTION

Early mammalian embryos contain cells that are capable of differentiating into the entire set of tissues that comprise the fetal and postnatal organism. Because of their wide differentiative capacities, these cells are called pluripotent stem cells. Ordinarily, complex interactions with specific neighboring cells in the intact developing organism guide the differentiation of the pluripotent stem cells into the intermediate progenitor cells of tissues and organs.

Embryonic stem (ES) cells are derived from preimplantation embryos and from cultured primordial germ cells (for reviews, see, Joyner (1993) Gene Targeting, a Practical Approach, Oxford University Press: Oxford; Donovan (1994) Curr. Top. Dev. Biol. 29:189-225). ES cell lines have the remarkable capacity to proliferate in culture without loss of their euploid chromosomal complement and to maintain pluripotency. When they are combined with a preimplantation embryo to form a chimera, ES cells resume normal development and are capable of contributing to all tissues within an organism, including the germ line, (Joyner (1993) supra; Pedersen et al. (1993) Targeted Mutagenesis in Mice: A Video Guide, Cold Spring Harbor Laboratory Press, NY; Hogan et al. (1994) Manipulating the Mouse Embryo, A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, NY, pp. 254-290; Wassarman & DePamphilis (1993) Methods Enzymol. 225:803-918). These properties have led to the extensive use of ES cells as vectors for introducing exogenous genes into the mouse germ line and, in particular, for ablating the function of specific

genes through homologous recombination or gene targeting (Joyner et al. (1993) supra).

It would be useful to provide human cells to patients for the treatment of human disease conditions. The ability to provide the appropriate human cell for disease treatment by, for example, transplantation therapy, requires the development of methods for deriving and propagating human pluripotent stem cells in culture, as well as methods for controlling and directing cell differentiation into the desired cell lineage.

Relevant literature

The human embryo develops from a group of undifferentiated cells into an organism with many specialized cells, tissues, and organs. During embryogenesis, the fates of cells becomes gradually restricted as they enter new developmental pathways. The fate of the inner cell mass (ICM) and trophectoderm has been described for the mouse (Gardner and Papaioannou (1975) in Early Development of Mammals, Cambridge University Press, Cambridge, pp. 107-132). This study found that the entire fetus and extra-embryonic mesoderm is formed from the non-endoderm cells of the ICM. The ICM of the primate blastocyst contains undifferentiated non-committed cells with the potential to enter a full range of developmental pathways. As these cells differentiate, they lose the capacity to enter developmental pathways that were previously open to them (Anderson (1992) Anim. Biotechnol. 3:165-175).

Embryonic stem cells, derived from preimplantation embryos (Martin (1981) Proc. Natl. Acad. Sci. 78:7634-7638; Evans & Kaufman (1981) Nature 292:154-156) and embryonic germ (EG) cells, derived from fetal germ cells (Matsui et al. (1992) Cell 70:841-847; Resnick et al. (1992) Nature 359:550-551) are undifferentiated, immortal cells capable of differentiating into derivatives of all three embryonic germ layers. Well-characterized ES and EG cells have been derived only from rodents. Pluripotent cell lines have been derived

from preimplantation embryos of several non-rodent species (Evans et al. (1990) Theriogenology 33:125-128; Graves & Moreadith (1993) Mol. Reprod. Dev. 36:424-433; Notarianni et al. (1991) J. Reprod. Fertil. Suppl. 43:255-260; Sukoyan et al. (1992) Mol. Reprod. Dev. 33:418-431), but the developmental potentials of these cells lines remains poorly characterized. Mouse ES cells remain undifferentiated through serial passages when cultured in the presence of leukemia inhibitory factor (LIF) and differentiate in the absence of LIF (Williams et al. (1988) Nature 336:684-687). Mouse ES cells combined with normal preimplantation embryos as chimeras and returned to the uterus participate in normal development (Bradley et al. (1984) Nature 309:255-256). Studies of factors that control *in vitro* differentiation have been reviewed by Pedersen (1994) Reprod. Fertil. Dev. 6:543-552. A recent study (Nakano et al. (1994) Science 265:1098-101) examined the role of CSF-1 in inducing myeloid differentiation of cultured mouse ES cells.

The mechanisms controlling differentiation of specific lineages have been studied with mouse ES cells grown *in vitro*. However, because of the significant differences between early human and mouse development, it is believed that human development cannot be accurately studied with mouse ES cells (Thomson et al. (1995) Proc. Natl. Acad. Sci. 29:7844-7848). For example, human and mouse embryos differ in the timing of embryonic genome expression (Braude et al. (1988) Nature 332:459-461), in the structure and function of the fetal membranes and placenta (Benirscheke & Kaufmann (1990) Pathology of the Human Placenta, Springer, New York), and in formation of an embryonic disc instead of an egg cylinder.

Human embryonal carcinoma (EC) cells, which are pluripotent, immortal stem cells from teratocarcinomas, have been induced to differentiate in culture (Andrews et al. (1984) Lab. Invest. 50:147-162), resulting in loss of specific cell surface markers and the appearance of new markers. The range of differentiation obtained from human EC cell lines is

more limited than that obtained from mouse ES cells, and varies widely between cell lines (Pera et al. (1987) Int. J. Cancer 40:334-343). All pluripotent human EC cell lines derived to date are aneuploid (Roach et al. (1993) Eur. Urol. 23:82-88, suggesting that EC cells do not provide an accurate representation of normal differentiation.

To date, no one has succeeded in establishing pluripotent cells from human embryos. A study conducted with rhesus monkey embryo-derived cells (Thomson et al. (1995) Proc. Natl. Acad. Sci. 29:7844-7848) documented the pluripotency of the derived cells. This cloned cell line remained undifferentiated and continued to proliferate more than 1 year in culture, maintaining a normal XY karyotype, and the potential to differentiate into trophoblast and to derivatives of embryonic endoderm, mesoderm, and ectoderm.

Recently, a paper by Bongso et al. (1994) Human Reproduction 9:2110-2117, describes efforts to derive such cells. Inner cell mass-derived cells were isolated and grown through two passages with the use of a human oviductal epithelial feeder layer in the presence of human leukemia inhibitory factor (LIF). The pluripotency of these cells was not characterized, nor was a substantial capacity for secondary culture of the cells demonstrated.

SUMMARY OF THE INVENTION

Methods and compositions are provided for the *in vitro* derivation and propagation of passagable pluripotent stem cells from early primate embryos. Such pluripotent stem cells differentiate into intermediate progenitor cell populations of a variety of cell types, including, hematopoietic, nervous system, muscle, and endodermal cells, through *in vitro* induction. The present invention describes methods for maintaining primate pluripotent stem cells in culture, and for controlling and directing the differentiation of such cells into a variety of cell types.

One aspect of the invention is a method for deriving passagable pluripotent stem cells from early embryos by a) growing an early primate embryo to the blastocyst stage under *in vitro* culture conditions in which the developmental potential of pluripotent stem cells is maintained; b) culturing blastocyst stage embryos on feeder cell layers of fibroblasts under conditions wherein cultured pluripotent cells are derived; and c) transferring the derived cells for propagation as secondary cultures. In a specific embodiment, such *in vitro* cultures utilize medium containing essential and non-essential amino acids and a defined protein source (which may include transferrin, insulin and LDL), in the absence of serum. A defined protein source is one of purified human or bovine serum albumin, or purified human or bovine serum non-albumin proteins.

An advantage of the invention is the *in vitro* production of passagable human pluripotent stem cells derived from early stage embryos, which are useful for the *in vitro* production of specifically desired cell types and lineages.

Another aspect of this invention is the treatment of a primate by administering cells of the invention to the primate.

Another aspect of the invention is an assay which exposes cells of the invention to a compound and determining the effect of the compound on development.

An advantage of the invention is that the cells can be maintained in culture for significant periods of time.

A feature of the invention is the cell culture medium comprised of a particular combination of ingredients.

These and other objects, advantages, and features of the present invention will become apparent to those persons skilled in the art upon reading the details of the compositions, composition components, methods, and method steps of the invention, as set forth below.

DETAILED DESCRIPTION

Before the methods and compositions of the present invention are described and disclosed it is to be understood that this invention is not limited to the particular methods and compositions described, and as such may, of course, vary. It is also understood that the terminology used herein is for the purposes of describing particular embodiments only, and is not intended to be limiting since the scope of the present invention will be limited only by the appended claims.

It must be noted that as used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural references unless the context clearly indicates otherwise. Thus, for example, reference to a "pluripotent stem cell" include multiple pluripotent stem cells.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any materials or methods similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing the particular information for which the publication was cited. The publications discussed above are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

Definitions

By the term **"stem cell"** or **"pluripotent stem cell"** which terms are used interchangeably, is meant cells that are capable of self-regeneration during propagation, and which

have the capacity *in vitro* or *in vivo* to differentiate and become intermediate stem cells that further proliferate and terminally differentiate into specific lineages. As used herein, "stem cells" refers to cells that have the potential for producing multiple cell types, including for example, hematopoietic, nervous system, muscle, or endodermal cells.

By the term "**embryonic stem cells**" is meant undifferentiated, immortal cells capable of differentiating into derivatives of all three embryonic germ layers. By the term "**passagable stem cell**" or "**passagable pluripotent embryonic stem cell**" which terms are used interchangeably, is meant stem cells, as described above, that have been propagated in an *in vitro* culture for a period of at least about one week, or through 3 or more passages, in an isolated form, *i.e.* in the absence of blastocyst-derived trophectoderm cells.

By the term "**propagation**" is meant to cellular reproduction. Generally the phenotype of the cell is maintained through propagation, including its potential for differentiation. The term also includes propagation into cultures where the cells are induced to differentiate into a particular lineage or lineages.

By the term "**early stage primate embryo**" is meant a multi-cellular structure derived by cleavage divisions of the zygote before implantation into the uterus.

By the term "**embryonic germ layer**" is meant either endoderm, mesoderm, or ectoderm cell layers, which are formed as a result of gastrulation and which interact in the formation of the bodily tissues and organs.

By the term "**blastocyst stage**" is meant a hollow ball stage embryo with an outer (termed the "trophectoderm") layer, and an inner group of pluripotent cells (termed the "inner cell mass").

By the term "**terminally differentiated cells**" is meant cells that comprise the predominant cell type of each organ or

tissue and have the morphological or biochemical properties that confer a unique identity to each tissue or organ.

By the term "**culturing**" or "**cell culture**" is meant the growth of embryos, cells, or tissue explants outside of the body in a tissue culture environment. Cell culture medium comprises salts and nutrients, *e.g.* essential and non-essential amino acids; and generally exogenous proteins, either in the form of defined proteins, *e.g.* purified human or bovine serum albumin; purified human or bovine serum non-albumin proteins; *etc.*, or an undefined protein source, *e.g.* fetal calf serum, human serum, *etc.*

By the term "**intermediate stem cells**" is meant stem cells that have a reduced capacity for differentiation relative to pluripotent stem cells, yet still retain the capacity for differentiation into one or more of the cell types contained within a particular tissue or organ.

By the term "**secondary culture**" is meant the propagation of explanted or derived cells after their initial placement in culture, demonstrating their sustained capacity for cell proliferation. The culture conditions may be the same as those used for the initial derivation, or may be altered to provide for differentiation of the cells.

By the term "**passaging**" is intended the process of transferring living cells, or colonies derived thereof, sequentially from one culture vessel to another with a concomitant increase in cell number.

By the term "**derived**" or "**deriving**" pluripotent stem cells is meant establishing the growth of ES cells in cell culture while maintaining their potential for differentiation, including self-regeneration during propagation, and capacity for further differentiation into multiple cell lineages. In the method of the invention, passagable pluripotent stem cells are derived in the absence of growth factors that induce differentiation, and in the presence of growth factors that maintain the cells in an undifferentiated state by restraining differentiation.

By the terms **"controlling"** and **"directing"** differentiation of pluripotent stem cells is meant directing the differentiation of pluripotent stem cells into specifically desired cell lineages through the control of culture conditions, including supplementing and depleting culture medium of specific growth factors.

By the term **"growth factor"** is meant a molecule capable of promoting growth or differentiation of cells, either in culture or in the intact tissue, through its specific effects on a transmembrane receptor. Growth factors include polypeptides, such as fibroblast growth factor, and non-polypeptide factors such as retinoic acid, that also specifically influence the growth and differentiation of cells through their action on intracellular receptor molecules that specifically mediate growth-promoting or differentiative effects.

By the term **"epidermal growth factor(s)"** is meant a factor that acts through an epidermal growth factor receptor. Known EGF receptors include EGF-R (HER-1), HER-2, HER-3, and HER-4. Known EGFs include epidermal growth factor, transforming growth factor α, heparin-binding epidermal growth factor, amphiregulin, heregulins, epiregulin, β-cellulin and cripto.

By the term **"fibroblast growth factor(s)"** is meant a factor known to act through a fibroblast growth factor receptor. Known FGF receptors include FGFR1, FGFR2 (including KGFR), FGFR3, and FGFR4. Known FGFs include FGF-1 (acidic FGF), FGF-2 (basic FGF), FGF-3, FGF-4, FGF-5, FGF-6, FGF-7 (keratinocyte growth factor), FGF-8 (androgen-induced growth factor), and FGF-9 (glial activating growth factor).

By the term **"inhibitor of the function of specific growth factor or receptor"** is meant an agent that interferes with the biological function of a growth factor, either by interacting with the factor itself, with its receptor, or by interfering with the function of other molecules that transduce the effects of growth factor-receptor interaction.

By the term "**inducing**" or "**induction**" of differentiation is meant causing the differentiation of cultured cells through certain additions, depletions, or other modifications of their environment, so as to bring about directed changes in the morphological or biochemical properties of the cells.

The terms "**treatment**", "**treating**", "**treat**" and the like are used herein to generally mean obtaining a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. "Treatment" as used herein covers any treatment of a disease in a mammal, particularly a human, and includes:

(a) preventing the disease or symptom from occurring in a subject which may be predisposed to the disease or symptom but has not yet been diagnosed as having it;

(b) inhibiting the disease symptom, i.e., arresting its development; or

(c) relieving the disease symptom, i.e., causing regression of the disease or symptom.

Embryonic Stem Cells

Passagable primate pluripotential embryonic stem cells are derived and propagated in *in vitro* culture. Methods are also provided for controlling the differentiation pathway of the passagable pluripotent stem cells to produce cells of a specifically desired type. These cells thus have significant clinical use in transplantation therapy for genetic disease, cancer, and other degenerative conditions, such as amyotrophic lateral sclerosis, Huntington's disease, Parkinson's disease,Alzheimer's disease, *etc.*

The derivation of primate pluripotent stem cells (inculding human pluripotent stem cells) is accomplished by isolating pluripotential stem cells from a blastocyst stage

embryo and growing the cells under conditions that maintain their viability and developmental potential. These conditions do not maintain the three-dimensional structure of the embryo itself or preserve its capacity for intrauterine development.

The derived cells may be passaged as secondary cultures for propagation of the pluripotent cells, or to induce differentiation wherein intermediate stem cells or terminally differentiated, tissue-specific cells are generated. Components of the *in vitro* culture medium, including nutrients, feeder layer cells, growth factors and protein source, are manipulated in order to achieve the maintenance of pluripotential capacity, or to drive the cells into differentiation. The culture medium and conditions are selected to optimize viability at each stage of the invention.

The source of pluripotent stem cells is the inner cell mass of a primate blastocyst stage embryo. Primate species of interest include humans; apes, *e.g.* chimpanzees, gorillas, orangutans and bonobos; old world and new world monkeys, *e.g.* macaques, baboons, *etc.*; and prosimians. When used, human embryos are obtained by donation with informed consent from patients, *e.g.* undergoing *in vitro* fertilization as therapy for infertility. Such donations adhere to protocols for human embryo research as approved by the responsible Institutional Review Board(s). The blastocysts may be grown in culture prior to isolation of the inner cell mass, or cells may be directly isolated from an *in vivo* derived blastocyst.

Where the intact embryos are cultured from early cleavage stages (*i.e.* preimplantation embryos), the medium comprises simple components. Suitable medium includes Earles' medium; Hank's balanced salt solution (HBSS); Eagle medium, Potassium simplex optimization medium (KSOM), *etc.* The medium will include essential amino acids, at a concentration ranging from about 0.5X to 5x the standard concentration, *i.e.* that which is typically found in medium such as Dulbecco's modified Eagle medium (DME). Non-essential amino acids are also

included, at a comparable concentration. The initial medium will further comprise a protein source, such Synthetic Serum Substitute (Irvine Scientific Co., Irvine, CA); purified human or bovine serum albumin; purified human or bovine serum non-albumin proteins, *e.g.* LDL, transferrin, insulin, *etc.* Preferably, the medium is not supplemented with a complex source of protein, *i.e.* it is essentially serum-free. Culture environmental components include extracellular matrix components, *e.g.* fibronectin, vitronectin, laminin, collagen type IV or Matrigel; low oxygen tension (<20%); antibiotics; and reducing agents, *e.g.* b-mercaptoethanol, monothioglycerol, *etc.*

Pluripotent inner cell mass cells are isolated from the trophectoderm by culture outgrowth, or by selective lysis of the trophectoderm. In the second approach, the trophectoderm cells are destroyed, leaving the inner cell mass. For example, the trophectoderm may be coated with a molecular layer of trinitrobenzene sulfonic acid, washing, treating with an antibody to its derivative, DNP, and then treating with active serum complement. The immunosurgery may also be accomplished by coating the trophectoderm with anti-species antibody (*i.e.* anti-human, anti-gorilla, *etc.*) followed by washing and treatment with active serum complement. Alternatively, lysis is accomplished by brief treatment with a Ca^{++} ionophore, *e.g.* A23187 at a concentration of 1-5 x 10^{-5} M, to lyse the outer cells. These processes release the inner cell mass, which is then cultured as isolated cells on a feeder cell layer, as described below.

Alternatively, the intact cleavage stage embryo is placed on the feeder cell layer and allowed to attach, flatten and grow as a two-dimensional structure containing the pluripotent cells at the center of the outgrowth. The pluripotent cells are isolated by physical separation, *e.g.* by micropipet. The culture conditions for blastocysts and isolated inner cell mass cells are the same unless otherwise specified, herein termed "derivation medium".

The derivation medium comprises a complex culture medium, *e.g.* Dulbecco's modified eagle medium (DME); RPMI; Iscove's modified Dulbecco's medium (IMDM); *etc.*; supplemented with non-essential amino acids and environmental culture conditions as previously described. It is not necessary to supplement with essential amino acids, which are already present in the medium formulation. A feeder layer is used. A number of different cells can be used as a feeder layer, *e.g.* fibroblasts derived human oviductal epithelium; fetal fibroblasts derived by primary culture from the same species as the embryo; buffalo rat liver cells (BRL) cells; *etc.* These cell layers provide non-defined components to the medium and restrain the differentiation of the pluripotent cells.

The derivation medium is deficient in differentiative growth factors, *i.e.* those that induce differentiation in embryonic stem cells, *e.g.* epidermal growth factor; fibroblast growth factor(s) and include factors known to act through an epidermal growth factor receptor, including the EGF-R (HER-1) and HER-2, HER-3, and HER-4, and/or a fibroblast growth factor receptor, including FGFR1, FGFR2 (KGFR), FGFR3, and FGFR4. In a preferred embodiment, serum-free medium is used, supplemented with defined, purified proteins, *e.g.* transferrin; albumin; insulin; *etc.* where the growth factors are absent *ab initio* from the medium. Alternatively, serum is used if the differentiative growth factors are depleted from it, for example by affinity chromatography, *etc.* The differentiative growth factors may also be present in the medium, but blocked from exerting their biological activity through the addition of blocking agents, *e.g.* antibodies that interfere with the binding of the factors and their cognate receptor; or alternatively with tyrphostins that specifically interrupt the signal transduction cascade mediated by epidermal growth factor receptor, thus impairing the effect of the several growth factors that act through the EGF receptor, *etc.*

The blastocyst culture medium further comprises at least one maintenance growth factor, *i.e.* factors that stimulate the growth of stem cells while maintaining their potential for differentiation. A cocktail comprising two or more maintenance growth factors may also be used. Specific maintenance growth factors include leukemia inhibitory factor (LIF), oncostatin M; IL-6/IL-6 receptor soluble complex; colony stimulating factor-1 (CSF-1), steel factor (c-kit ligand), and ciliary neurotrophic factor (CNTF). The use of LIF is of particular interest. Generally, maintenance growth factors are supplied in a concentration range of about 100-10,000 U/ml. Optimum concentrations for each growth factor can be readily determined by one skilled in the art.

After the blastocyst is grown in culture for five to ten days in derivation medium, the inner cell mass expands into a small cluster of pluripotent cells and their differentiated derivatives. These passagable pluripotent stem cells are isolated mechanically, dissociated slightly, *e.g.* in trypsin, EDTA, dispase collagenase, *etc.*, and transferred to fresh feeder layers for further culture, using the conditions described for derivation medium. Where trypsin is used for passaging the cells, the medium may be modified to include trypsin inhibitors, *e.g.* peptide substrates, serum, soybean trypsin inhibitor, *etc.*

The passagable pluripotent embryonic stem cells thus derived have a number of uses, including differentiation into multiple cell lineages, drug screening, as a model for differentiation, *etc.*

The subsequent differentiation into intermediate stem cells or terminally differentiated cells is achieved through exposure to differentiative growth factors, including erythropoietin, bone morphogenetic protein(s), epidermal growth factor(s), and fibroblast growth factor(s), and to culture environmental components including the extracellular matrix components fibronectin, vitronectin, laminin, and collagen type IV, low oxygen tension (< 20%), and reducing

agents. The concentration of each supplemented factor or component will depend on the specific factor or component, and may be readily determined by one skilled in the art. For example, fibroblast growth factor(s) is supplied in a concentration range of approximately 10-100 ng/ml. Such cultures may utilize medium that is depleted in stem cell maintenance growth factors, including human LIF, CNTF, CSF-1, steel factor, *etc.*

The intermediate stem cells of the invention find use as therapeutic agents by transplantation to regenerate lineages of a host deficient in stem cells. Conditions where such therapy is used include rescuing a subject that is diseased, *e.g.* suffering from lymphoma, leukemia, or other neoplastic condition, and can be treated by removal or destruction of bone marrow and hematopoietic tissue by irradiation or chemotherapy, followed by engraftment with the cells of the invention.

The cells of the invention may be used for the treatment of genetic diseases, by restoring genetic function in genetically modified somatic cells. For example, diseases including, but not limited to, β-thalassemia, sickle cell anemia, adenosine deaminase deficiency, recombinase deficiency, recombinase regulatory gene deficiency, *etc.* may be corrected by introduction of a wild-type gene into the cells of the invention, either by homologous or random recombination. Other indications of gene therapy are introduction of drug resistance genes to enable the cells of the invention to have an advantage and be subject to selective pressure during chemotherapy. Suitable drug resistance genes include, but are not limited to, the gene encoding the multidrug resistance (MDR) protein. Diseases other than those associated with hematopoietic cells may also be treated, where the disease is related to the lack of a particular secreted product including, but not limited to, hormones, enzymes, interferon, growth factors, or the like. By employing an appropriate regulatory initiation region, inducible production

of the deficient protein may be achieved, so that production of the protein will parallel natural production, even though production will be in a different cell type from the cell type that normally produces such protein. It is also possible to insert a ribozyme, antisense or other message to inhibit particular gene products or susceptibility to diseases.

The passagable pluripotent stem cells may be frozen at liquid nitrogen temperatures and stored for long periods of time, being thawed and capable of being reused. The cells will usually be stored in 5% DMSO and 95% fetal calf serum. Once thawed, the cells may be expanded by use of growth factors or stromal cells associated with stem cell proliferation and differentiation. The cells may optionally be modified to have at least one non-autologous gene, as described above for use in gene therapy, and such genetically altered populations and their progeny are embraced within the scope of this invention.

Pharmaceutical preparations are also provided, comprising the cells of the invention in a form suitable for administration, *e.g.* by injection or infusion, to a patient in need thereof, in combination with a suitable carrier medium for use in any of the foregoing treatments. Also provided are the cells of the invention for pharmaceutical use, and use of the cells of the invention in the manufacture of a pharmaceutical preparation, *e.g.* for use in any of the foregoing treatments.

Dosages of the cells of the invention for pharmaceutical uses such as reconstitution of the hematopoietic system of a patient in need thereof, will vary depending on the nature of the condition to be treated and the other aspects of the patient's treatment, *e.g.* prior radiation or chemotherapy, or co-therapy with agents having an influence on hematopoiesis, *e.g.* cytokines, as well as on the purity and viability of the cell population to be administered.

The cells of the invention are also of use as research tools in producing various lineage restricted cell lines;

detecting and evaluating growth factors relevant to stem cell self-regeneration; assaying for factors associated with development; and to test drugs that modulate or affect such systems and treat or alleviate such diseases as Parkinson's disease; to replace tissue destroyed by degenerative or autoimmune disease; *e.g.* b-islet cells; identifying support cells (endothelial, stromal, fibroblast) in the developing embryo that allow for growth and differentiation of various lineages, *etc.* Thus, the stem cells of the invention may be used in assays to determine either autocrine or paracrine regulatory signals and evaluate responses to growth factor either from external or intrinsic protein sources; and to determine the activity of media, such as conditioned media, evaluate fluids for cell growth activity, involvement with differentiation into particular lineages, or the like.

Stem cell factors may be isolated from media or cell extracts of supportive cells in which a population of the cells of the invention are growing or a supernate of a population of the cells, by separating or fractionating the fluid, *e.g.* chromatographically. The active fraction containing the desired factor is identified by measuring the growth and differentiation of stem cells in the presence and absence of such fractions, or alternatively, using comparative analysis of fluid obtained from a population of stem cells. Additionally, cDNA libraries of the cells of the invention may be prepared and screened for genes encoding factors of interest. Growth factor or receptor genes in the cDNA libraries may optionally be amplified and identified using oligonucleotide primers based on conserved sequences within known growth factor or receptor families.

Immortalized cells of the invention are useful as being cells that are responsive to a factor allowing for the regeneration of stem cells, *e.g.* in assays for survival, activation, or proliferation in the presence and absence of the putative stem cell growth or maintenance factor.

EXAMPLES

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to establish human pluripotent stem cell lines and their induced differentiation, and are not intended to limit the scope of what the inventors regard as their invention. Unless indicated otherwise, parts are parts by weight, temperature is in degrees centigrade, and pressure is at or near atmospheric pressure. Efforts have been made to ensure accuracy with respect to numbers used (e.g., molecular weights, amounts, particular components, etc.), but some deviations may exist.

Example 1

Establishment of Human Pluripotent Stem Cell Lines

Pre-blastocyst culture conditions. Generally, embryos fertilized *in vitro* or retrieved from the reproductive tract at early cleavage stages are cultured to the blastocyst stage at 37oC in an atmosphere of 5% CO_2, 95% air, in a simple medium such as KSOM (See Lawitts and Biggars (1993) Meth. in Enzym. **225**:153-164) supplemented with a protein source, such Synthetic Serum Substitute (Irvine Scientific Co., Irvine, CA), purified human or bovine serum albumin protein, purified human or bovine serum non-albumin proteins (including transferrin, LDL, insulin, *etc.*), and with essential amino acids at 0.5x-5x their concentration in Dulbecco's modified Eagles medium (DME), and non-essential amino acids at comparable concentrations (see Ho *et al.* (1995) Mol. Rep. and Develop. **41**:232-238).

While the nutritional requirements of early (preimplantation) stage primate embryos are simple, consisting primarily of three-carbon sugars or glucose, the purity of all reagents, particularly water, is essential. The protein source is provided for ease of handling and the amino acids are provided as a fixed nitrogen source.

Blastocyst culture conditions. When embryos reach the blastocyst stage (or at the eight cell stage for human embryos, in the event that they are available before the blastocyst stage), they are transferred to a complex medium, such as DME. DME is supplemented with non-essential amino acids, β-mercaptoethanol or monothioglycerol, nucleosides, and antibiotics as previously described (Robertson (1987) in Teratocarcinomas and Embryonic Stem Cells: A Practical Aproach, Oxford University Press, Oxford, UK, pp. 71-112). Additional supplements include the growth factors described below under "Growth factors added during derivation and propagation of pluripotent stem cells."

Feeder cell layer conditions. Fibroblasts, or fibroblast-like cells grown as monolayers, are instrumental in the derivation of pluripotent primate cells. Fibroblasts from a variety of sources can be used for this purpose, for example, mouse fetal or human fetal fibroblasts, or human oviductal epithelium cells. For use, the feeder cells are inactivated by irradiation with 5-6,000 rads, 250 kVp, or treatment with 0.01 mg/ml mitomycin C for 3.5 h at 37°C; then they are plated, washed, and grown to confluency on gelatin or extracellular matrix-coated dishes (as described below). The feeder layers are then used for growing the early stage embryos or secondary stem cell cultures.

Growth factors added during derivation and propagation of pluripotent stem cells. The following polypeptide growth factors are added as supplements to the DME medium during pluripotent stem cell derivation and propagation to maintain the pluripotent, undifferentiated state of the stem cells which would otherwise differentiate in their absence. The cytokines leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) are used at 100-10,000 U/ml. The cytokine macrophage colony stimulating factor 1 (CSF-1) is used to sustain the proliferation of the pluripotent stem cells and is present at a concentration of between 100-1000 U/ml. Other growth factors are oncostatin M at a

concentration of from 2 to 200 ng/ml, usually about 20 ng/ml; and IL6 at a concentration of from 50 to 5000 ng/ml, usually 500 ng/ml; IL6 soluble receptor at a concentration of from 1 to 10 mg/ml, usually 5 mg/ml.

Other factors in stem cell derivation. The key novel aspect of deriving pluripotent cells from early stage primate embryos lies in restraining their spontaneous differentiation into the early lineages and embryonic germ layers during post-blastocyst culture. This is accomplished by a combination of biophysical, biochemical, and biological conditions.

Biophysical conditions. The effects of oxidative damage on the survival of pluripotent cells is restrained by maintaining cells at below ambient oxygen levels (20%), achieved by mixing nitrogen with air to achieve the necessary lower O_2 levels (5-10%). In addition, small amounts of a reducing agent are consistently maintained in the culture medium (β-mercaptoethanol, 0.2-1.0 ppm (v/v); monothioglyceral).

Biochemical conditions. The substrata on which pluripotent cells are cultured have a role in deterring differentiation of pluripotent cells. Accordingly, during derivation of pluripotent stem cells from early stage embryos, the embryos and secondary cultures are maintained on extracellular matrices, either those produced by the feeder cell layers (see below), gelatin, or purified matrix components, such as fibronectin, vitronectin, laminin, and/or collagen type IV, or MATRIGEL™ (Collaborative Research, Inc.) In the latter instance, culture dishes are coated with extracellular matrix component(s) by placing a solution (20-40 μg/ml) in the dish for 2 h, then washing the dishes with saline before adding culture medium, and embryos or cells to the dishes. Feeder layer-derived extracellular matrices are generated by culturing non-inactivated fibroblasts to confluency on dishes, then lysing the cells with Triton X-100 (0.5% v/v), and washing extensively with saline.

Growth factor depletion strategies. Key to propagating primate pluripotent cells in an undifferentiated state is to deplete the growth medium of factors that induce their differentiation into intermediate stem cells or terminally-differentiated cells. In a preferred embodiment, serum-free medium is used to achieve essentially complete absence of differentiative growth factors.

Alternatively, serum may be depleted of differentiation inducing substances. Specifically, epidermal growth factor(s) and/or fibroblast growth factor(s) are depleted from the serum used in the derivation of pluripotent cells from early stage embryos. This is accomplished either by using a defined protein source (Synthetic Serum Substitute, Irvine Scientific Co.), by selecting serum types and specific lots low in these factors, or by extracting the specific growth factor from the serum. For example in the latter case, fibroblast growth factors are removed from serum by stirring DME medium containing 20% serum overnight in the presence of an equal volume of heparin-derivatized Sephadex beads, which bind fibroblast growth factors and remove them from the medium. If necessary, the depletion step is repeated until the growth factor(s) are present at below threshhold levels for inducing stem cell differentiation. In the case of other growth factors, removal is accomplished by derivatizing Sephadex beads with an antibody to the growth factor, mixing beads with serum-containing medium, and repeating this treatment until the differentiation-stimulating activity is removed from the serum. Once the pluripotent stem cells are derived (i.e., for propagation in secondary culture and thereafter), the growth factor-depleted serum may be replaced with complete serum, *e.g.* 10% heat-inactivated fetal bovine serum and 10% heat-inactivated newborn calf serum, which have been quality control tested for the capacity to support the secondary culture of established embryonic stem cells.

Functional deprivation strategies. An alternative to depleting the growth medium of differentiation-stimulating

growth factors is to functionally interfere or perturb the activity of the growth factors. Where a specific function-perturbing antibody is available, the action of specific growth factors is inhibited by adding the function-perturbing antibody directly to the serum-containing medium to inactivate the factor. Where a growth factor receptor function-perturbing antibody is available, the antibody is added to the medium, thereby blocking the function of the specific growth factor receptor.

An alternative to functional interventions, the signal transduction process initiated by binding of a polypeptide ligand to its transmembrane receptor is interrupted by specific inhibitors. For example, epidermal growth factor receptor (EGF-R) transduction is interrupted by treating the cells with certain tyrphostins that are EGF-R selective tyrosine kinase inhibitors. Similarly, the action of FGF signalling receptors is inhibited at specific steps of the Ras/mitogen-activated protein (MAP) kinase pathway. As with growth factor depletion, functional deprivation is discontinued after derivation of pluripotent stem cells (i.e., in secondary cultures and thereafter) provided that the undifferentiated phenotype of the particular stem cell persists without the functional perturbation.

<u>Biological conditions</u>. Feeder cell layers are prepared as described above and used as an adjunct to the defined factors that support derivation and secondary culture of pluripotent stem cells. Regarding the role of the feeder cell layers, no rigorous distinction can be made between provisions of undefined growth factors to the medium, or removal by exhaustion or adsorption of growth factors that would otherwise induce differentiation of the stem cells.

<u>Induced differentiation of pluripotent stem cells</u>. The general method for inducing the differentiation of established pluripotent stem cells into intermediate stem cells and subsequently into terminally differentiated cells is related to the derivation strategy in that it relies on a

deprivation/supplementation regimen involving specific polypeptide growth factors. To induce differentiation of the pluripotent stem cells, it is first necessary to release the inhibition conferred by maintenance growth factors such as LIF, CNTF, *etc.* This is accomplished by omitting the maintenance growth factors from the culture medium. Simultaneously, exposure to one or more, but not all, polypeptide growth factors is used to induce the specific differentiation of intermediate stem cells. For example, fibroblast growth factor (10-100 ng/ml) is added to induce the differentiation of mesoderm and its derivatives, such as muscle. Other growth factors are omitted or specifically depleted to achieve specificity of differentiation, and thus prevent induction of undesired productions (e.g., induction of the myeloid pathway by CSF-1). The methods for achieving the depletions and/or functional deprivations are the same as those described above.

Example 2.

Pluripotent stem cell culture in serum-free medium.

Murine pre-implantation embryos were tested for the outgrowth of ES cell lines, *i.e.* cell lines that show the same morphology and proliferation rate as ES cell lines with demonstrated germ-line capacity. Blastocysts were grown in Knockout-DMEM™ containing ES cell supplement serum replacement (Life technologies R&D, Grand Island, NY). The results are shown in Table 1.

TABLE 1

medium	mouse strain	embryos plated	growth after first passage	ES cell lines
serum-free	C57/B6, CBA	12	12	10
serum	C57/B6, CBA	12	12	1
serum-free	C57	41	37	19
serum	C57	41	24	4

The data in Table 1 suggest that serum-free conditions improve the outgrowth of ICM-derived cells, and the derivation of ES cell lines.

Human pre-implantation embryos were grown in Earle's medium supplemented with essential and non-essential amino acids and with synthetic serum supplement. The embryos were then moved onto KSOM with BRL cell feeder layers supplemented with essential and non-essential amino acids and with synthetic serum supplement. After 5 to 7 days total growth past-fertilization, the blastocyst stage embryos were transferred to two different culture conditions.

The serum-free cultures contained mouse fibroblast feeder layer cells, DME, 25 mM sodium bicarbonate, 1X non-essential amino acids, 2% bovine serum albumin, 200 mg/ml transferrin, 10 mg/ml insulin, 20 mg/ml LDL, 1% penicillin/streptomycin and 7.5 x 10^{-5} M monothioglycerol. As maintenance growth factors, both LIF (100 U/ml) and oncostatin M (22 ng/ml) were added to the medium.

The serum containing cultures contained DME with 10% fetal calf serum and 10% newborn calf serum and LIF at 100 U/ml.

Cultures were evaluated after 5-7 days. The results are shown in Table 2, where outgrowths are defined as a blastocyst that has attached and spread in two dimensions to a

feeder layer of cells and is showing cell proliferation.

TABLE 2

type of medium	# human blastocysts	# outgrowths
serum	8	0
serum-free	16	11

These data indicate that the outgrowth of pluripotent stem cells from a human blastocyst is strongly dependent on the presence or absence of differentiative growth factors in the culture medium, and that the use of serum-free medium provide a substantially complete absence of such differentiative factors.

What is claimed:

1. A method of deriving and maintaining passagable primate pluripotent stem cells, said method comprising the steps of:

a) isolating inner cell mass cells from a primate blastocyst;

b) culturing said inner cell mass cells in medium comprising: a feeder cell layer of cells, a defined protein source, at least one maintenance growth factor, and containing essential and non-essential amino acids; wherein passagable primate pluripotent stem cells are derived from said inner cell mass cells.

2. A method according to Claim 1, wherein said primate is a human.

3. A method according to Claim 1, wherein said medium is serum-free.

4. A method according to Claim 1, wherein said defined protein source comprises one or more of purified human serum albumin protein, bovine serum albumin protein, transferrin, insulin, or serum non-albumin proteins.

5. A method according to Claim 1 said maintenance growth factor is one or more of colony stimulating factor-1, steel factor, leukemia inhibitory factor, ciliary neurotrophic factor, oncostatin M, and IL-6/IL-6 receptor soluble complex.

6. A method according to Claim 1, wherein said inner cell mass cells are derived from a blastocyst cultured *in vitro*.

7. A method according to Claim 1, wherein said inner cell mass cells are generated by selective lysis of trophectoderm cells.

8. A method according to Claim 1, further comprising the step of transferring said passagable primate pluripotent stem cells to a secondary culture in medium comprising a differentiative growth factor.

9. A method of claim 1, further comprising:

(c) maintaining the cells in culture with pluripotent potential from a period of time of x days or more.

10. An *in vitro* cell culture, comprising:

medium comprising a feeder cell layer of cells, a defined protein source, at least one maintenance growth factor, and containing essential and non-essential amino acids; and

passagable primate pluripotential embryonic stem cells.

11. An *in vitro* cell culture according to Claim 10, wherein said primate is a human.

12. An *in vitro* cell culture according to Claim 10, wherein said medium is serum-free.

13. A method of assaying compounds, comprising:

providing cultured passagable primate pluripotent stem cells;

contacting the cells with a compound to be tested; and

observing the effect of the compound on the cells.

14. A method of treatment, comprising:

a therapeutically effective amount of cultured passagable primate pluripotent stem cells.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/10316

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N5/06 C12N5/00 G01N33/50 C12N5/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 12665 A (DIACRIN INC) 11 May 1995 see page 1, paragraph 3 see page 3, paragraph 3 - page 5 see page 14 - page 15 ---	1,4-10, 14
X	WO 90 01541 A (AMRAD CORPORATION LTD) 22 February 1990 see page 4, line 7 - page 5, line 27 see page 12, line 20 - page 13, line 7 --- -/--	1,2,5, 10,11

[X] Further documents are listed in the continuation of box C.

[X] ~~Patent family members are listed in annex.~~

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 October 1997	11. 11. 97

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Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	De Kok, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/10316

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THOMSON J A ET AL: "ISOLATION OF A PRIMATE EMBRYONIC STEM CELL LINE" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol. 92, no. 17, August 1995, WASHINGTON US, pages 7844-7848, XP000604992 cited in the application see the whole document, esp. page 7848, column 1	1,4-10
Y		2,11,14

Y	BONGSO A ET AL: "ISOLATION AND CULTURE OF INNER CELL MASS CELLS FROM HUMAN BLASTOCYSTS" HUMAN REPRODUCTION, vol. 9, no. 11, 1994, OXFORD GB, pages 2110-2117, XP000613489 cited in the application see the whole document	2,11,14
A		1,4-6, 8-10

A	WO 95 16770 A (ABS GLOBAL INC) 22 June 1995 see the whole document	1-14

A	NICHOLS J ET AL: "DERIVATION OF GERMLINE COMPETENT EMBRYONIC STEM CELLS WITH A COMBINATION OF INTERLEUKIN-6 AND SOLUBLE INTERLEUKIN-6 RECEPTOR" EXPERIMENTAL CELL RESEARCH, vol. 215, no. 1, November 1994, US, pages 237-239, XP002044063 see the whole document	1,3,5

A	US 5 453 357 A (HOGAN BRIGID L M) 26 September 1995 see column 9	1,5

P,X	WO 96 22362 A (WISCONSIN ALUMNI RESEARCH FOUNDATION) 25 July 1996 see the whole document	1,4-10, 13,14

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Inter onal application No
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INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claim(s) 14
are directed to a diagnostic method
practised on the human/animal body , the search has been carried out and
based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 97/10316

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		EP 0770125 A	02-05-97